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STRUCTURAL CHARACTERIZATION  
OF THE PUTATIVE CHOLINERGIC BINDING REGION  $\alpha$ (179-201)  
OF THE NICOTINIC ACETYLCHOLINE RECEPTOR  
PART I. REVIEW AND EXPERIMENTAL DESIGN

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April 1993

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13. ABSTRACT (Maximum 200 words) Because of the complex nature of the nicotinic acetylcholine receptor, it has not been possible to develop a reliable model of the cholinergic binding site. For example, the receptor has a molecular weight over 250,000 daltons, five homologous subunits, and essential membrane bilayer. Small peptides of the region 170-210 of the $\alpha$ -subunit of the receptor have been shown to bind cholinergic agonist and competitive antagonist with binding profiles equivalent to the whole $\alpha$ -subunit and comparable to the whole solubilized receptor. It is likely that the folded structure of these peptides is similar to the folding of this region <u>in vivo</u> , and to some degree, is determined by the hydrophobic interaction of the residues within the relatively short region of $\alpha$ (170-210). By reducing the investigation primarily to this region, a considerably simpler model may provide a practical quantifiable model for understanding the underlying process of cholinergic binding at the molecular level. The research proposed investigates the structure of the putative cholinergic binding region of the nicotinic acetylcholine receptor and the contribution of the lipid bilayer membrane to cholinergic binding.				
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## PREFACE

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1. BACKGROUND

The nicotinic acetylcholine receptor (AChR) is an essential component of the complicated mechanism of signal transmission at the neuromuscular junction (fig 1). At the neuromuscular junction nerve impulses are transmitted, via the chemical neuro-transmitter acetylcholine (ACh), from the pre-synaptic membrane of the nerve cells to the post-synaptic muscle membrane. The AChR is an integral transmembrane protein with agonist binding sites located on the synaptic side of the muscle membrane (DeFeudis 1974). The transmembrane domain forms a cation specific ion channel through the muscle membrane. Acetylcholine binds to the AChR initiating the ion-channel gating response which mediates sodium cation flux across the cell membrane as a signal to initiate muscle contraction (Neher & Sakmann 1976).

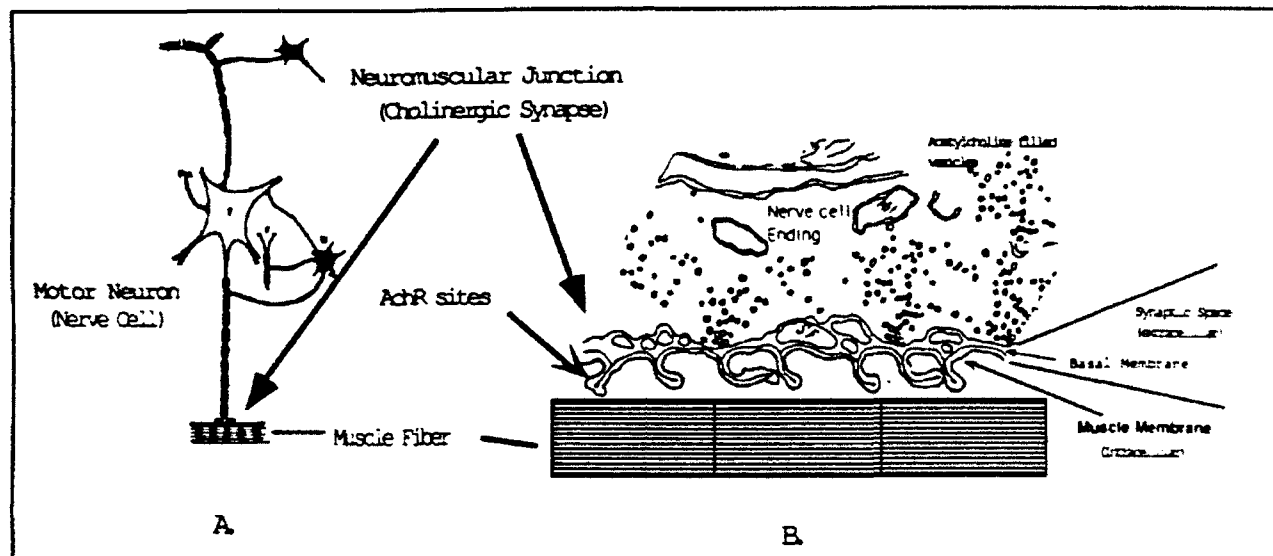


Figure 1. A. Motor neurons and muscle at the neuromuscular junction, B. Schematic drawing of motor endplate showing neuromuscular junction and the synaptic space between the nerve ending and muscle. Junctional folds of the synaptic cleft extend into the muscle, high concentrations of acetylcholine receptors are found in the folds. Redrawn from Bell, G.H., et al., 1980.

In spite of the proliferation of refereed reports on the acetylcholine receptor, over 500 from 1986-1990, a high resolution model for the cholinergic binding region of the receptor does not exist. In the absence of direct measurement of the tertiary structure, such as atomic coordinates from X-ray crystallography or multidimensional data from NMR spectroscopy, the tertiary structure must be inferred from knowledge derived from the primary sequence, selective proteolytic digest, affinity binding and labeling studies and weakly inferred properties such as secondary structure prediction, hydrophobicity and antigenic site mapping. Recently, the use of site-directed mutagenesis has provided additional information for elucidation of the tertiary structure of the acetylcholine receptor.

The AChR is a pentameric, transmembrane glycoprotein composed of two  $\alpha$ -subunits and one each of  $\beta$ ,  $\gamma$  and  $\delta$  subunits giving a stoichiometry of  $\alpha_2\beta\gamma\delta$  (Reynolds & Karlin 1978, Lindstrom et al 1979, Raftery et al. 1980). The AChR has two functional regions, the ligand-gated ion channel and the cholinergic binding sites (fig 2). Topographically there are three domains: (i) the synaptic (extracellular) domain that reportedly contains most of the protein mass, (ii) the cytoplasmic (intracellular) domain and (iii) the transmembrane domain that consists predominantly of  $\alpha$ -helices from each subunit that traverse the membrane multiple times forming the ion-channel.

Using electron microscopy, electron diffraction and helical image reconstruction methods Toyoshima & Unwin (1990) resolved the overall structure to 17 Å and reported dimensions of the AChR as roughly 125 Å along the axis perpendicular to the cell membrane (the cylindrical axis), with a diameter of ~70 Å as viewed on end with a central pit of 25-30 Å. They found the extracellular portion extends approximately 65 Å beyond the cell surface and makes up approximately 55% of the protein mass. The cytoplasmic end of the receptor extends approximately 20 Å into the cytoplasm and contains approximately 20% of the mass, the transmembrane portion is about 30 Å long and is about 25% of the mass (Toyoshima and Unwin 1990; Toyoshima and Unwin, 1988; Brisson 1986; Giersig et al. 1986). The circular arrangement of subunits around the central ion-channel is shown in fig 2c. (Brisson 1986, Giersig et al 1986, Toyoshima and Unwin 1988).

The primary sequence of subunits from muscle, neuronal and ganglia type AChR, have been determined by cDNA sequencing for many species (table 1). Noda et al (1982) first reported the  $\alpha$ -subunit of *Torpedo californica* followed by the sequences of the other three subunits (Noda et al 1983a). Noda and coworkers reported 19% complete

identity of amino acid residues for all four subunits and 20% identity for three out of four subunits. In the three out of four identity set, 54% of the amino acid substitutions were chemically conservative replacements. There is a high degree of homology among the  $\alpha$ -chains of these proteins, for example the molecular weights of T. californica, human and calf AChR  $\alpha$ -chain are 50,116, 49,694 and 49,897 respectively. The degree of homology is 97% for human/calf, 81% for human/T. californica, and 80% for calf/T. californica (Noda et al. 1983b). The amino acid sequence of the  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ -subunit of T. californica is given below in table 2.

Cysteine residues are conserved at positions 128, 141, 192 and 193 in the  $\alpha$ -subunit, and it is currently believed that disulphide bonds are formed between Cys128-Cys141 and Cys192-Cys193 (Karlin 1969, Karlin 1980, Koa et al. 1984). Criado et al. (1986) proposed that sequence  $\alpha$ 127-143 is probably not fully exposed on the surface of the muscle membrane, and Cys127-Cys142 probably form a disulfide bond that is not the disulfide bond reported to be close to the cholinergic binding site (Karlin 1969, Karlin 1980, Koa et al. 1984). Oblas et al. (1986) provided evidence using proteolytic fragments that residues Cys128-Cys142 form a disulfide bond. While results of Neumann et al. (1986) indicate neither Cys128 nor Cys142 form stable disulfide bonds with Cys192 or Cys193. The specific nature of cysteine participation in disulfide bonds is still uncertain, but for the present most of the literature indicates that Cys128-Cys142 and Cys192-Cys193 participate in disulfide bonds (Dennis et al. 1986, Kao et al. 1984, Dennis et al. 1988 and Langenbuch-Cachat et al. 1988) and Cys192-Cys193 are in the proximal area of the cholinergic binding site (Karlin 1983). In spite of the general acceptance of the constraint that cysteines 192, 193 are in the proximity of the cholinergic binding site, as demonstrated by affinity labeling, the stoichiometry of labeling with 4-(N-maleimido)-benzyltrimethylammonium iodide (MBTA) is only 1:1 (MBTA:AChR), not 2:1 as would be expected (see below), and can only be demonstrated in Dithiothreitol (DTT) reduced receptor.

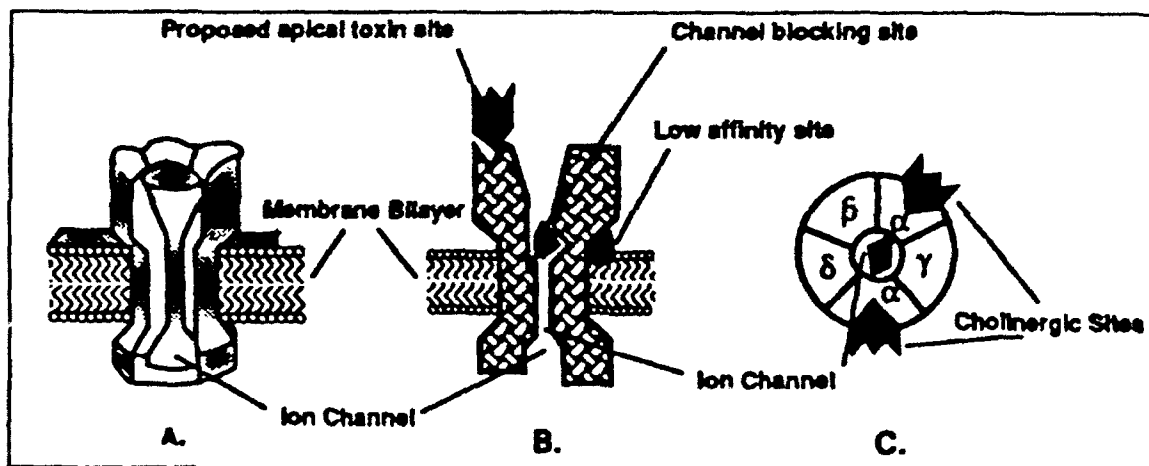


Figure 2. A. Schematic model of the pentameric AChR showing the proposed transmembrane topology. B. shows the low affinity site, channel blocking site and apical  $\alpha$ -toxin and agonist site (the agonist site is still debated). C. top view of the circular arrangement around the ion channel. (Redrawn from Changeux et al. 1984).

Potential glycosylation sites, consensus Asn-Xaa-Ser/Thr, were found in the  $\alpha$  and  $\beta$  subunits at residues 169<sup>1</sup>; in the  $\gamma$ -subunit at residues 96, 169, 343, 486; and in the  $\delta$ -subunit at 96, 169, 236 and conserved across species at  $\alpha$ Asp141<sup>2</sup> (Maelicke 1988). Poulter et al. (1989) reported that by mass spectrometry all four subunits are glycosylated at consensus numbered residue 143<sup>3</sup> ( $\alpha$ -141) by high mannose type oligosaccharides, and the  $\delta$  and  $\gamma$  subunits contained complex type oligosaccharides. On the  $\alpha$  and  $\beta$ -subunits the only possible site of N-glycosylation is Asp141, therefore the region surrounding it is probably on the extra-cellular surface of the receptor, and lies in a  $\beta$ -region between two predicted  $\beta$ -sheet structures possibly forming a stable, tightly packed motif (Noda et al. 1983). This region is also predicted to be hydrophilic by the Kyte & Doolittle (1982) method. The precursor  $\alpha$ -subunit mentioned above has a 24 amino acid sequence that is probably a secretory signal sequence (Anderson et al. 1983; Noda et al. 1982), and is present on all subunits.

It was proposed that the acetylcholine binding site is formed by the sequence  $\alpha$ -127-143 (Noda et al. 1982), but in comparison with the other subunits  $\beta$ ,  $\gamma$ ,  $\delta$  which apparently do not bind acetylcholine, the sequences (127-143) are well conserved (table 3). The conservation of these residues and the lack of evidence of binding on the other chains would indicate this may not be the binding site. Smart et al. (1984) proposed that the substitution of a Gln139 residue in the  $\alpha$ -subunit for Trp139 in the other subunits produced folding and charge patterns sufficiently different to provide for the specific binding of cholinergic ligands. Criado et al. (1986) demonstrated the sequence 127-143 is probably not involved in the formation of the binding site for ACh, using a synthetic peptide and anti(127-143)-peptide

Footnotes: 1. 169 is a consensus number for all four aligned immature subunits. 2. 141 is Asn in the mature  $\alpha$ -subunits. 3. 143 is the consensus number for the mature, aligned  $\alpha$ -subunits of several species.

1	100
α MLCSTYHVGILVLLFSCCGIVGSEZTRIVANLL-EN-YN-RVIRPVKHHTKFDITVGLQLIGLISVDE-VNQIVeTHVFLRQWIDVRLVWNPACT	
β MENVIRMAIGLVVMAIALSIVGASVMEDELISVLF-ET-YNPRV-TPAQTVGDRVVFVGLTLTNLLSINERIEE-HTTHVFLNLAHIDYRLQWNPACT	
γ MVL---CILLIICL-ALeVr---SeNEEGRLLIKLL-qD-YDRKII-PARTLDNIIDVTURKLTYNLLISINER-EALITTHVFLZIQWADYRLQWNPACT	
δ NQNHIFVYLLIscLYSGCS---GVNEERLLINOLLIVNRYNKHV-RPVKHANVVMIALSLTSLNLSLKE-CDETLSVWQDCHAMyDRLQWNPACT	
101	200
α GGIRKIRLPSSDDVWLPDLVLYNNAOGDFaIVNHTLLLDyTGRINWLPFAIFRSyCeILVTNPPFDQGNCTNRIGINTYDGTVEISa-----PESCRP	
β EGIRKIRLPSSDDVWLPDLVLYNNDGSPFITIHVNVLVQHTGAYSA---FADIRSSCTIRVHYTFPDMQNCNTHVRSYTYTSEVTQO---NALANGERE	
γ EGIDLVIRIPSELLMLPDVVLNNHVDGQFEVATYAMVLVYUGSHYVLPFAIYRSTCPISVYTFPDMQNCNTHVRSYTYTSEVTQO---NALANGERE	
δ SDISILRLPPELVNIPDVLQNNNDGQYVATYGVVLPVNGYVTLPPAIFRSYCPINVLVTFPDMQNCNTHVRSYTYTSEVTQO---NALANGERE	
201	300
α -----DLSFNEaGEWVNRdyrGwKINVYtccpD-TPYIDITTHINQRIPLYTVVWVVIIPCLLESFLTGLVFLYLPDSCG-EKHTLSISVLLSLTVFL	
β VREIVINADAFTEGQMSIEHQPSRQW----RSDDPSTEDVTFFYLIIQRKPLFYIVYCIIPCLISILAILVFLYLPDAG-EKHTLSISVLLSLTVFL	
γ VEMINIDPEFTENGENTIRHAPAKQYTHQIKR-DDTDFQELIFFLIIQRKPLFYIINIIPCVLISILVVLVFLYLPDAG-EKHTLSISVLLSLTVFL	
δ IEMIIDPEFTENGENTIRHAPAKQYTHQIKR-DDTDFQELIFFLIIQRKPLFYIINIIPCVLISILVVLVFLYLPDAG-EKHTLSISVLLSLTVFL	
301	400
α LVIVE-LIPaTsSAVPLIGKYLPLMIFVLSIIII--TVVIVNHNKSPSTHTHPQWVRKIFIDTIPNVHES-----SKP:LS	
β LLIAQKV-PETSLVPIITRYLNFIMILVASSVIL--SVVVLNHNKSPSTHTHPQWVRKIFIDTIPNVHES-----SKP:LS	
γ LLIAQKV-PETSLVPIITRYLNFIMILVASSVIL--SVVVLNHNKSPSTHTHTPNTHTSPKIFIDTIPNVHES-----SKP:LS	
δ LLISQRL-PETALAVPLIGKYLPLMIFVLSIIII--TVVIVNHNKSPSTHTHTPNTHTSPKIFIDTIPNVHES-----SKP:LS	
401	500
α RAEKQENKIFADD-IDISD-----ISGRQV---TGEVIFq-----TPLIKHPD-----VKSATIEGVRYIAENMRSGEEaMAA-EE---MYTVAN	
β RAEKQENKIFADD-IDISD-----ISGRQV---TGEVIFq-----TPLIKHPD-----VKSATIEGVRYIAENMRSGEEaMAA-EE---MYTVAN	
γ RAEKQENKIFADD-IDISD-----ISGRQV---TGEVIFq-----TPLIKHPD-----VKSATIEGVRYIAENMRSGEEaMAA-EE---MYTVAN	
δ RAEKQENKIFADD-IDISD-----ISGRQV---TGEVIFq-----TPLIKHPD-----VKSATIEGVRYIAENMRSGEEaMAA-EE---MYTVAN	
501	600
α VIDHILLCVPLIC-IIGTVSVF-AGRIEELSQ---EG-----	
β VADRILLYTVFVIC-SIGTISIFLDAshNVPPDPP-A-----	
γ VIDR-ACFWIALLESIGTLAIFLTKHFNQVPEFPDGI-KYVP-----	
δ VIDR-ACFWIALLESIGTLAIFLTKHFNQVPEFPDGI-KYVP-----	

Table 2. Amino acid sequences of  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  subunits of *Torpedo californica* with proposed transmembrane regions, M1-M4 and disulfide bonds. The numbering is for the immature sequence prior to cleavage of the signal peptide. Favored substitutions are upper case, others are lower case. Gaps have been inserted in the sequence to achieve maximum homology (Noda et al. 1983b). \* - the glycosylation site of the  $\alpha$ -subunit (Noda et al. 1982).

monoclonal antibodies. However, Atassi et al. (1987) demonstrated five toxin binding sites including residues 127-143 using synthetic peptide fragments and monoclonal antibodies.

Neumann et al. (1986) mapped the  $\alpha$ -bungarotoxin binding site to a 15kDa proteolytic fragment that initiates beyond  $\alpha$ -Asp152 and terminates in the region of Arg313/Lys314, and demonstrated the sequence 185-196 binds  $\alpha$ -bungarotoxin directly, this binding was completely inhibited by d-tubocurarine. Aronheim et al. (1988) and Gershoni (1988), further characterized this area by demonstrating a bacterially expressed (*E. coli*, -Trp) cDNA fusion protein, TrpE(a184-200), binds with  $\alpha$ -bungarotoxin and has a pharmacological profile qualitatively reflecting binding by the intact receptor, and the sequence  $\alpha$ 197-200 contributes a point of contact between the receptor and the protein neurotoxin  $\alpha$ -bungarotoxin.

Most studies indicate there are two binding sites per AchR monomer (Maelicke 1984; Popot and Changeux 1984), this is consistent with the binding site being located in the  $\alpha$ -subunits. The two  $\alpha$ -chains reportedly contain most

<i>Torpedo californica</i>	Noda et al. (1982)
<i>Torpedo marmorata</i>	Devillers-Theiry et al. (1983)
Mouse	Boulter et al. (1985)
BC3H-1	Merlie et al. (1983)
Ox	Noda et al. (1983b)
Human	Noda et al. (1983b)
PC12	Boulter et al. (1986)
Xenopus	Baldwin et al. (1988)
Calf	Noda et al. (1983b)
Chick	Ballivet et al. (1983)
Drosophila	Bossey et al. (1988)

Table 1. c-DNA primary sequences of the n-AchR  $\alpha$ -subunit for several species. (partial list)

possibly all of the binding region for cholinergic agonists and competitive antagonists, and may provide for allosteric control of the receptor function. Haggerty and Forehner(1981) reported SDS-denatured AchR  $\alpha$ -subunits, and not the other subunits, uniquely bind  $\alpha$ -bungarotoxin as the first true demonstration of the toxin binding activity of the  $\alpha$ -subunit.

Numerous reports have shown the region of residues 170-210 of the  $\alpha$ -subunit can be affinity labeled(Kao et al.1984, Dennis et.al,1986) or can independently bind  $\alpha$ -neurotoxins such as  $\alpha$ -bungarotoxin (Neumann et.al,1985, 1986 a,b; Wilson et.al.1985; Mulac-Jericevic & Atassi, 1986; Pedersen et.al. 1986; Barkas et.al.1987, Gershoni 1987) with reasonable affinity,  $K_d = (0.7 - 350) \times 10^{-8}$  (Neumann, et al 1986a; Gotti et. al. 1987; Lentz et.al. 1987; Aronheim et.al. 1988; Wilson & Lentz, 1988). The more traditional model of cholinergic binding(Kosower, 1989) is described as a cationic site, followed by a hydrophobic patch that interacts with the methylene groups of acetylcholine, followed by an anionic site that has an electrostatic interaction with the quaternary amine of acetylcholine(fig 3a). This anionic site was originally thought to be an aspartic acid, but recently evidence has been presented proposing that the phenolic hydroxyl of  $\alpha$ -Tyr198 ionizes to provide the anionic site(Hawrot 1991). Since then Tomaselli et.al. (1991) expressed site-mutated AchR in *Xenopus* oocytes and identified  $\alpha$ -Tyr190 as critical to cholinergic binding and  $\alpha$ -Tyr198 mutated to phenylalanine produced "small but significant" effects.

Stauffer and Dougherty(1989) proposed a model analogous to the proposed binding of phosphocholine to the monoclonal antibody McPC603(fig 3b). The binding of phosphocholine was based on the X-ray crystal structure of the active site of the monoclonal antibody, and is depicted as the ion-dipole interaction of the quaternary amine of phosphocholine with the  $\pi$ -system electrons of the aromatic amino acid residues. In this model they demonstrated the positive charge of the guest(quaternary amine) is "solvated" by electron-rich faces of aromatic rings of the hosts receptor using synthetic cyclophane style hosts(fig 3c). Although, the ion-dipole model does not rule out contributions from the earlier model, all of the cholinergic binding can be accounted for on the basis of the ion-dipole model.

The requirement for a membrane lipid bilayer to support the acetylcholine receptor is well documented(for review see Barrantes 1989). The requirement of a membrane, or at least the contribution of the membrane to cholinergic binding is far less certain. Behling and Jelinski(1990) suggested that acetylcholine first binds to the membrane and assumes a conformation that is recognized by the receptor and diffuses rapidly through the membrane to the receptor. Rhodes et.al(1985) calculated that for model systems the approach of a ligand to a receptor by diffusion in the lipid bilayer is about 1000 times faster than diffusion through the aqueous medium. Adam and Delbruck(1986) demonstrated that the predicted maximum reaction rate was increased if the ligand was restricted to a two dimensional surface containing the target site, thus reducing the translational states available to the reactants.

Noda et.al(1983) suggested that because of the considerable sequence homologies, all subunits of the AchR should have fundamentally similar secondary and tertiary structures. The N-termini of the subunits are probably on the extracellular side of the membrane as demonstrated by Anderson et.al.(1981,1982,1983), even though the N-termini sequences are not bound by antibodies in the native receptor(Neumann et.al. 1984; Ratnam & Lindstrom 1984). Anderson's criteria were first, signal recognition protein was required for integration of the  $\delta$ -subunit into microsomal membranes, second the signal sequence is cleaved on integration into dog pancreatic microsomal

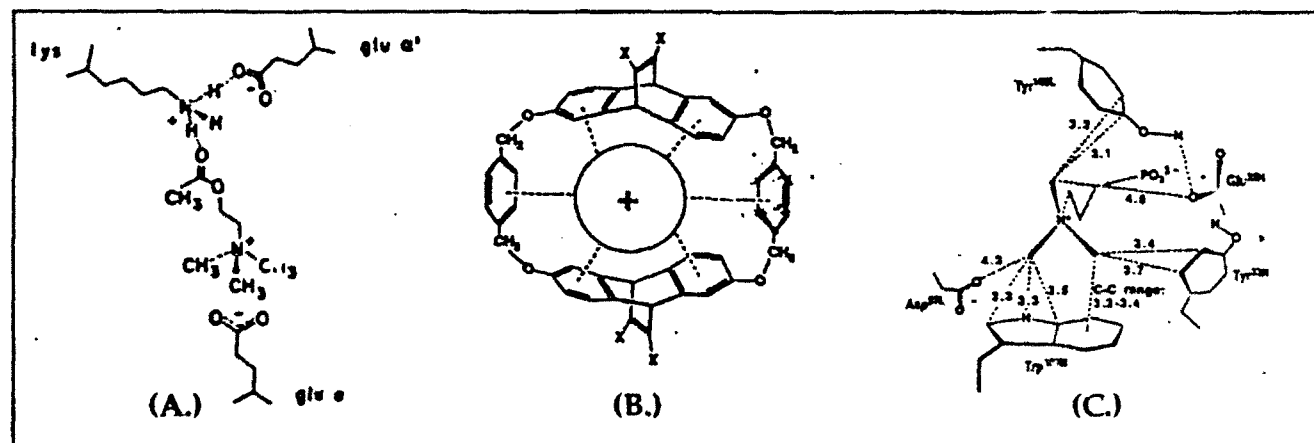


Figure 3. (a.) Proposed model for cholinergic binding constructed from three amino acid side chains Kosower(1989), (b.) Phosphocholine McPC603, close contacts (in angstroms) between the carbons of the N-methyl groups and other heavy atoms are shown along with selected hydrogen bonds. A large number of contacts < 3.5 Å are made to the aromatic residues tyr<sup>198H</sup>, tyr<sup>198L</sup>, and especially Trp<sup>107H</sup>. One of the N-methyl groups lies almost directly over the center of the benzene ring of Trp<sup>107H</sup>, with six C-C contacts in the 3.2 to 3.4 Å range. Satow, et. al.(1986) (c.) The stabilizing, cation-p interaction is symbolized by dotted lines between the positive charge of the ammonium and the p faces of the aromatic rings that define the interior of the host. Stauffer and Dougherty(1989)

membranes, and finally the partially determined NH-terminal sequence of the cleaved subunit is the same as the known sequence of the mature native  $\delta$ -subunit. Several groups (Lindstrom et al., 1984; Young et al. 1985; Ratnam et al. 1986a,b) describe the carboxy termini to be on the cytoplasmic side as determined by antibody mapping. This would imply an even number of transmembrane crossings by the subunits, thus excluding several models proposing odd numbers of crossings. There is currently no conclusive evidence demonstrating the orientation of the carboxy termini with respect to the membrane. The N-terminal extra-cellular portion of the  $\alpha$ -subunit is reported to extend from the N-terminal residue Ser(1) to Ile(210) of the mature subunit (Claudio et al. 1983; Noda et al. 1982; Devillers-Thiery et al. 1983). However, Criado and coworkers (1985) and Lindstrom and coworkers (1984) respectively proposed, based on antibody mapping studies, that amino acids 152-159 and 159-169 are on the cytoplasmic side of the membrane thus the N-terminal extra-cellular portion could only extend from residue 1 to approximately residue 125.

	1	1	1	2
	2	a.	8	b.
	8	2	4	0
$\alpha$	CEIiVTHFFFDQNC		WKHwVYtccPD-TPYIDITY	
$\beta$	CTIKVMYFFFDWQNC		RKNW-RSDDPSEYEDVTF---	
$\gamma$	CPiAVTTFFFDWQNC		KKNYnWqltK-DDtDFQEIiF	
$\delta$	CPInVLYFFFDWQNC		KKNi-YpdkfPNgTNYQDVTF	

Table 3. (a.) Comparison of sequences of *T. californica* subunits 128-143, (b.) Comparison of *T. californica* sequences 184-204. Favored substitutions are upper case, others are lower case. Gaps have been inserted in the complete sequence to achieve maximum homology. From (Noda et al 1983b).

After alignment of the four subunits of the AchR extensive topological homologies become apparent. Noda et al (1982), from analysis of hydrophobicity and secondary structure predictions, proposed four transmembrane helical segments labeled M1 - M4 (fig 4a, see Table 1). Claudio et al. 1985, and Devillers-Thiery et al. (1983) also proposed theoretical models with four helical transmembrane segments that result in the N-terminal and C-terminal on the synaptic side of the cell. Fairclough et al. (1983) and Young et al (1985) proposed a five transmembrane helical model (fig 4b) as did Guy (1983) using partition energy and helical packing analysis and Finer-Moore (1984) using amphipathic analysis. Both Guy (1983) and Finer-Moore (1984) described the fifth transmembrane region as an

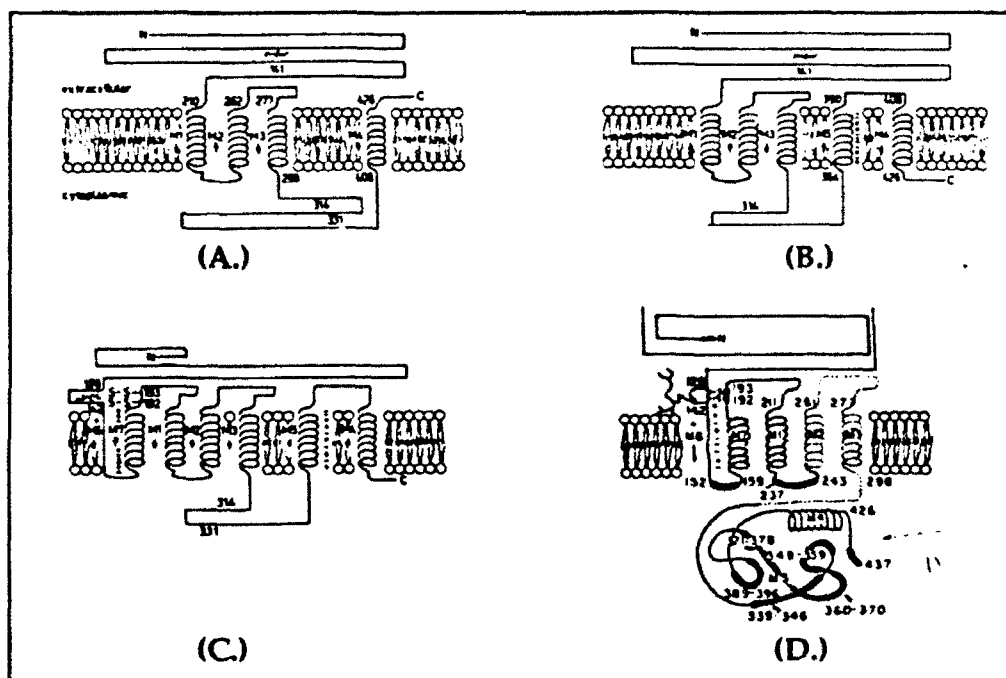


Figure 4. Models of AchR topography with respect to the membrane bilayer. The 4-helical model (A) was proposed by Noda et al. (1982), Claudio et al. (1983), and Devillers-Thiery et al. (1983). The 5-helical model (B.) was postulated by Fairclough et al. (1983), Guy (1984), and Young et al. (1985). The 7-transmembrane domains (C.) were proposed by Criado et al. (1985). Ratnam et al. (1986) proposed a model referred to as the "bulky cytoplasmic domain" model (D.). Redrawn from Barrantees (1989).

amphipathic helix that provided the interface between the hydrophobic transmembrane helices and the polar channel lining. Criado et.al.(1985) using antibody mapping methods demonstrated a potential for seven transmembrane helices(fig 4c) with M6 and M7 transmembrane segments between the N-termini and M1. Ratnam et.al. (1985) developed a model based on a "bulky" cytoplasm domain with five transmembrane segments(fig 4d).

Moore et.al.(1974) reported the secondary structure content of the AchR by circular dichroism(CD) to be 34%  $\alpha$ -helix, and 29%  $\beta$ -structure. Mielke et.al.(1984) and Aslanian et.al.(1983) likewise reported the following structural contents by CD and Raman studies: 20-35%  $\alpha$ -helix, 30-50%  $\beta$  structure and 30-40% random coil. In view of the consensus that the ion channel is made up of transmembrane  $\alpha$ -helices(Guy 1984, Maelicke 1987), the percent  $\alpha$ -helix content of the N-terminal is probably lower than these numbers indicate. In fact, secondary structure analysis by Chou & Fasman(1978) algorithm for just the extra-cellular,  $\alpha$ -chain predicts only 17%  $\alpha$ -helix content.

The purpose of the ion channel is to provide a permeable pathway through the membrane for cations. Most of the models for transmembrane crossings as described above predict that each subunit contributes one transmembrane  $\alpha$ -helical subunit to form the membrane region of the ion channel (Dani et al 1989). There continues to be controversy about whether the channel "pore" lining is made from uncharged helices or highly charged amphipathic helices (Guy & Hucho 1987). Most reports favor the uncharged lining, including permeation studies by Dani & Eisenman (1987), and a classical Nernst-Planck continuum theory model by Levitt(1991). The channel has been described as having a wide mouth facing the synaptic space, tapering to a narrow region facing the interior of the cell (Dani 1989). The synaptic entry to the ion channel, distal to the pore entry(fig 5) is described by Toyoshima & Unwin(1990) as approximately 25Å in diameter, but the diameter reduces sharply at the membrane region of the ion channel to less than 10Å. The narrowest part of the channel being at the cytoplasmic side near the end of the membrane bilayer, possibly where the ion-gating function is centered. The opening of the channel at the cytoplasm, as in the opening at the extracellular region, sharply increases from the membrane region outward into the cytoplasm.

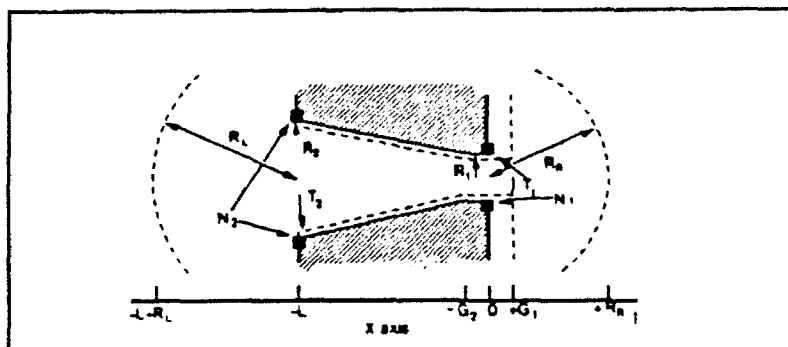


Figure 5. Cross-section through the model of the AchR channel. The solid lines outline the electrostatic channel and the dashed lines outline the physical channel. The narrow end of the channel faces the cell interior. The solid squares indicate sections through the ring of fixed charge of valence  $N_2$  and  $N_1$ . The channel is drawn to scale for the following dimensions (in angstroms): lipid thickness ( $L = 32$ ); electrostatic radius at narrow and wide end ( $R_1 = 4$ ;  $R_2 = 10$ ); physical radius at narrow and wide end ( $T_1 = 3$ ;  $T_2 = 9$ ); length of narrow electrostatic region ( $G_2 = 4$ ); length of extension of narrow region beyond electrostatic region ( $G_1 = 4$ ). From: Levitt (1991).

Rings of fixed negative charge are placed at the inner and outer surfaces of the membrane bilayer and these charges correspond to the known charges at the ends of the M2 segment(Imoto 1986). In addition to showing the essential negatively charged residues at the ends of the M2 transmembrane helix regions by substitutions of neutral and positive charges for negatively charged amino acids, Imoto et. al.(1986) used T. californica/Bovine chimera channels to demonstrate the presence of the M2 segment in the channel pore lining. The conductance of the T. californica AchR is greater than conductance for the bovine AchR. By constructing chimeras of the T. californica receptor with a bovine M2 segment and a bovine receptor with a T. californica M2 segment they demonstrated that it is the M2 segment that determined the magnitude of conductance. Using intra-channel non-competitive antagonist Giraudet et al (1986) and Hucho et al (1986) found these compounds labeled the M2 segment providing further evidence that the uncharged M2 segment forms the primary channel lining.

Karlin et al(1986) and Dani(1989) suggest the M1 segment is just behind the M2 segment in the channel pore. The M1 helices are labeled by the non-competitive antagonist quinaquine, and it is only found to label M1 when the channel is open(Karlin 1986). Also the M1 region is highly conserved, and a proline is conserved in all ligand-gated channel receptors including the GABA and glycine receptors in addition to all the AchR types. This proline has been implicated as the flexible region that participates in pore opening and closing.

The objective of this work is to characterize the region  $\alpha(179-201)$  of the AchR of *Torpedo californica* by direct physical measurements and interpret the results in terms of the molecular mechanism of cholinergic binding. In order to compare the structure of the peptides in different environments the experiments will be performed first in buffer solutions then with the peptides in lipid bilayer vesicles. Vibrational spectroscopic methods, both Raman and Fourier Transform Infra-Red (FTIR), will be used to measure changes in the secondary structure due to binding of cholinergic agonist and antagonist. Ultraviolet (UV) resonance Raman and absorption difference spectroscopy will be used to investigate the local environment of aromatic residues and the conformation of tryptophan residues in this peptide. UV spectroscopic, second derivative and difference spectra methods will be used to study the exposure of the aromatic residues to solvent, and to titrate the phenolic hydroxyl of the tyrosine residues. Finally, the topography of the peptide will be examined using mass spectrometry to identify proteolytic fragments of the membrane bound cholinergic peptide. The 23-amino acid peptide  $\alpha(179-201)$  will be the primary molecule studied, and spectra from this fragment will be compared to spectra of the whole  $\alpha$ -subunit and 12 kDa or 15 kDa proteolytic fragments of the subunit.

Salient features of the peptide  $\alpha(179-201)$  (Fig. 5) are vicinal cysteines at residues 192-193 which reportedly form a disulfide bond (Kao & Karlin 1986), six aromatic residues four tyrosines and two tryptophans and seven charged polar residues, two lysines, one arginine, one histidine and three aspartic acids. Other than theoretical predicted secondary structure, there are no reports describing experimental estimates of the secondary structure of this region.

## 2.1 Vibrational Spectroscopy

### *FTIR and Raman effect measurements of the peptide backbone structure*

FTIR and Raman effect spectra are physically different techniques which measure of the normal mode vibrations of a molecule (Wilson et al 1955). FTIR spectroscopy measures the absorbance of infra-red radiation, where as Raman spectroscopy measures inelastic scattering of monochromatic light, usually visible. Used together FTIR and Raman effect spectra can yield complementary information about the structure and conformation of a molecule. The vibrational spectrum of a molecule is a result of both the three dimensional structure and the vibrational force field. Even so, the structure of molecules can rarely be obtained directly from a vibrational spectrum. Important inferences about changes in conformation, however can be obtained from vibrational spectroscopy. From an analysis of N-methylacetamide Miyazawa et. al. (1958) determined normal mode vibrational frequencies that correspond to a general guide for initial assignment of peptide vibrational group frequencies which is usually followed by more rigorous experimental or theoretical assignment. The spectra of peptides is generally more complex than a simple summation or combination of the constituent amino acids because of coupling between nearby groups, and in the case of peptide bonds the vibrational bands are directly affected by hydrogen bonding that is characteristic of secondary structure types.

In extended repeating groups of protein secondary structure types, for example each turn of an  $\alpha$ -helix, the perturbations of groups of these normally isolated peptide bond chromophores can be seen as frequency and intensity shifts of the spectra. These changes which, are characteristic of secondary structure types of peptide vibrational bands, arise from the group modes of the secondary structure types (Krimm and Bandekar 1986). For example, the C=O—H-N hydrogen bonded pair are aligned with the cylindrical axis of an  $\alpha$ -helix, and the repeat of this bond and its contribution to the modification of the N-H stretch and C=O stretch vibrational bands can be interpreted quantitatively as the percent of helical structure. Hydrogen bonds shift the energy of the three peptide vibrations, the C=O stretch and N-H stretch are lower in energy because the hydrogen bond facilitates the stretch of the C=O toward the donor and the N-H stretch toward the acceptor. The primary bands used for assignment of the peptide backbone are the N-H stretch at  $\sim 3300\text{ cm}^{-1}$ , Amide I C=O stretch at  $\sim 1630-1690\text{ cm}^{-1}$ , Amide II (IR active) N-H deformation at  $\sim 1520-1550\text{ cm}^{-1}$  and Amide III a more complex mode that also depends on the side chain moiety. Each peptide bond contributes to these and other bands characteristic of protein secondary structure types (Krimm and Bandekar 1986). The Amide II band shifts to higher energy because the hydrogen bond makes the deformation of the N-H bond more difficult. Likewise  $\beta$ -structures such as parallel and anti-parallel pleated sheets have characteristic Amide I type vibrational bands.

The extent of change in secondary structure as measured by shift and intensity changes in amide bands (Table 5) will be compared to changes in these vibrational regions when cholinergic antagonist are bound to the peptide. Spectra of the bound peptides will be collected in the presence of excess antagonist succinylcholine and carbamylcholine. Experimentally measured spectra will be analyzed by the methods of Fong and McNamee (1987) for FTIR and Yager

et al(1984) for Raman. Results of this analysis will be used to determine what types of conformational change, if any, occurs when the peptide binds to cholinergic ligands. The Chou & Fasman predicted structure of the peptide indicates that the peptide is expected to be a combination of  $\beta$ -sheet and  $\beta$ -turn structures, and the experimental results will be compared to these predicted structures.

#### UV resonance Raman of aromatic residues

Resonance enhancement of the Raman vibrational spectrum of a chromophore permits selective measurement of that chromophore even in the presence of considerable background. By using monochromatic light, of a frequency close to the electronic transition energy of a chromophore, the polarizability at that frequency is dominated by that chromophore, then Raman scattering involving vibrations localized in that chromophore will be sharply enhanced (Cantor and Schimmel 1980).

UV resonance Raman using exciting light at 240 nm show bands at 600-1700  $\text{cm}^{-1}$  which derive from the tyrosyl and tryptophan residues (Miura et al 1988, 1989, Asher et al 1991). These spectra can be used to titrate tyrosine residues and to measure the conformation of tryptophan residues. The strength of hydrogen bonds at N-1-H of the tryptophan indole ring, or the conformation of the indole ring relative to the amino acid backbone, observed by Miura et. al. (1989) are reflected in frequency shifts of resonance Raman Bands. The two indole ring vibrations at 1487-1496  $\text{cm}^{-1}$  (W4) and 1422-1441  $\text{cm}^{-1}$  (W6) increase in frequency with increasing strength of hydrogen bonding at the N-1-H site. W6 has a large linear frequency variation with hydrogen bonding. Bands at 1422  $\text{cm}^{-1}$  indicate there is no hydrogen bonding and at 1441  $\text{cm}^{-1}$  indicate strong hydrogen bonding. The indole ring vibration W3 (1557-1542  $\text{cm}^{-1}$ ) changes frequency as a function of torsion angle  $\phi$ , defining orientation of the indole ring with respect to  $C_\alpha$ . The range of the shift is large enough to determine  $\phi$  from 60° to 120°, and represents the torsion angle from  $C_2$  of the indole to  $C_\beta$  then to  $C_\gamma$  and  $C_\delta$ .  $C_\delta$  moves away from  $C_\gamma$  as the W3 frequency increases. Miura et. al. (1988) reported success with this approach in structural studies of the side groups of lysozyme and  $\alpha$ -lactalbumin at frequencies 880  $\text{cm}^{-1}$  and 1340  $\text{cm}^{-1}$ , but these frequencies were difficult to assign because of overlap with amide group frequencies or NH and COOH terminal substituents (Miura et al 1988, 1989). It is because of the difficulty assigning bands at 880 and 1340  $\text{cm}^{-1}$  that bands W4, W6 and W3 were chosen as alternatives.

## 2.2 Electronic Spectroscopy, Second Derivative, and Difference Methods

From studies with small peptides,  $\alpha$ (170-186), Pearce et.al.(1990) proposed that tyr198 ionizes to provide the anionic site for binding the quaternary amine of cholinergic agonist. Subsequently Tomasselli et. al.(1991) found replacement of Tyrosine(198) with Phenylalanine had "small but significant effects" on cholinergic binding, but replacement of Tyrosine(190) with Phenalanine caused complete loss of binding capability, these studies used whole receptors expressed in *Xenopus* oocytes. In both cases the tyrosine residues of this region participate in cholinergic binding.

There are four tyrosine residues in the cholinergic binding fragment  $\alpha$ (179-201), and spectrophotometric titration can demonstrate if the four tyrosine residues are equivalent or if the residues are in different micro-environments (Wetlaufer 1961). The shift of UV absorbance intensity from 275 nm to 295 nm due to ionization of the phenolic hydroxyl of tyrosine is well known (Wetlaufer 1961). This phenomena will be used to measure the degree of ionization of tyrosines in the synthetic peptide. The relation of absorptivity with acidity will yield a titration curve that, ideally, permits selective study of the protonation of individual tyrosine residues. Since the phenol of tyrosine has only two microscopic species (phenol and phenolate) the apparent ionization constant ( $pK'$ ) can be calculated as follows:

$$pK' = pH - \log[a/(1-a)]$$

$$a = (e - e_0) / (e_1 - e_0)$$

$e$  = molar extinction coefficient at some  
fractional degree of ionization  $a$ .

$e_0$  is the molar extinction coefficient when  $a = 0$ .

$e_1$  is the molar extinction coefficient when  $a = 1$ .

The ionization of the tyrosine residue 190 or 198 to form the anionic portion of the binding site, will be investigated by measuring UV second derivative spectra. The spectra of the native peptide will be measured at a series of pH's ranging from pH 3.0 to 12.4, and the changes of the spectra, including time dependence and reversibility, will be monitored. Absorbance changes that are time-dependent or irreversible often indicate denaturation of the peptide.

Ragone et al(1984) used second derivative UV spectroscopy to determine the degree of exposure ( $a$ ) of aromatic residues to solvent based on the differential solvent effects on the absorbance of tyrosine. The second

derivative of the spectra follows Beers Law and can effectively resolve overlapping bands of the normal mode vibrations. Absorbance minima centered at 283 and 290.5nm and maxima centered at 287 and 295nm were used to calculate absorbance differences. Ratios(*r*) of the peak to trough distances in the second derivative spectra were calculated from these maxima and minima values, and this value related the degree of tyrosine exposure to the solvent and is dependent on the tyrosine to tryptophan content of the protein. The ratio(*r*) is calculated using the following formulae.

$$r = DA_1''/DA_2''$$

$$= (A''_{287} - A''_{283})/(A''_{295} - A''_{290.5})$$

$$= (Ax + B)/(Cx + 1)$$

for DA1'' and DA2'' equal to the second derivative differences at the stated wavelengths. The constants A, B, C correspond to:

A = De''1(Tyr)/De''2(Trp), B = De''1(Trp)/De''2(Trp) and  
C = De''2(Tyr)/De''2(Trp) and De'' = the difference between the second derivative of the molar extinction coefficients of the indicated pairs of fixed wavelengths and x = the molar ratio of Tyr/Trp(Ragone et. al. 1984)

Mode	Secondary Structure Types				
	$\alpha$ -helix	$\beta_{\text{antiparallel}}$ pleated sheet	$\beta_1$ Type turns	$\beta_2$	$\beta_3$
Amide I		[1701] 1695	1690		1686
	[1663] 1659	1669		1666 ~1656	
	[1650]	1630	-1640		1646±3
Amide II		[1587] 1555	1575 1558	1558	1562
	[1546] 1543			1547	1551
		1534	1536	1540	1539
	1520	1523			
Amide III			1324 1305	1329 1303	1317 1303
	1287 1270		1299	1297	1291
		1247 1228 1225			
Amide V	[639] 628	[709] 713	595 575	644 607	671 589
	609	702	574	594	577
	[589]	[699]	570	588	573
				572	573
					571

Calculated Amide Mode Frequencies for Various Structures Values in brackets represent frequencies not observed  
Rewritten from Krimm and Bandekar(1986)

The ratio(r) can be used to calculate the percent contribution of tyrosine and tryptophan residues. Equimolar N-acetyltyrosine amide and N-acetyltryptophan amide will be used as reference spectra for the calculation of difference and derivative spectra to determine the degree of exposure of tyr and trp residues under native conditions.

### 2.3 Mass Spectrometric Sequencing of Proteolytic Fragments

The location of the cholinergic binding site in relation to the lipid membrane is not known. If the sequence  $\alpha(170-210)$  is the main cholinergic binding region, is it located completely exposed in the synaptic space, is it in or partially in the membrane as suggested by Behling and Jelinski(1990), or is it folded within the rest of the protein. In order to better understand the environment of the peptide  $\alpha(179-201)$ , experiments will be performed to measure the partitioning of the peptide into the lipid bilayer membrane of small vesicles. If it is demonstrated that the peptide partitions into lipid bilayer membranes with consistent orientation, retention of activity (as measured by  $^{125}\text{I}$  binding) and efficiency(Rhodes et.al. 1985) then cleavage of the peptide by proteases or other methods will be used to determine if segments of the peptide are exposed and available for cleavage or protected from cleavage.

MS mapping and sequencing experiments of this type have been performed on complete reconstituted receptors(Poulter 1989, Moore et. al. 1989). Moore et.al.(1989) did not report sequences from 126 through 314 of the  $\alpha$ -subunit, likewise Poulter et. al.(1989) did not report sequences 173 through 314. The M1 proposed transmembrane helix is believed to start at about residue 210, however some investigators report the transmembrane helix starting closer to residue 228. This still leaves the location of the putative cholinergic site with respect to the membrane in question.

Peptide containing lipid vesicles will be prepared and characterized as described by McNamee et. al. (1986). Proteolytic digestion will be performed as described by Moore et. al. (1989). Fragmentation of the peptide in the membrane will be compared to fragmentation patterns of the peptide in buffer without the membrane. The peptide has six potential chymotrypsin cleavage sites, one trypsin, and one Staphylococcus V8 protease site. Mapping and sequencing will be performed as described by Fenselau(1991).

If the peptide completely inserts into the membrane and retains activity, this will be an indication that the in vivo environment is probably hydrophobic. Subsequent attempts at proteolysis may yield small peptide fragments, which will indicate that some of the membrane peptide is exposed and available for cleavage. However, if the peptide does not partition into the membrane it will suggest that the in vivo environment is hydrophilic.

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